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TITLE: Effect of Chemical Mutagens on Herpes Virus -  
Induced Cellular Transformation and Testing  
for Mutagenesis in Mouse Cells

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## ABSTRACT

Assays which detect quantitative increases of morphological transformation in 3T3 cells were employed to detect transformation by seven temperature sensitive mutants of herpes simplex type 2 virus. It was determined that all seven mutants caused transformation at the non-permissive temperature. Mutant A8 (293) caused formation of the most foci and appeared to be a more sensitive indicator of transformation in comparative experiments (31 foci per million cells compared to four foci per million cells for ultraviolet-irradiated wild type virus).

Mutagenesis experiments using THO cells as indicator cells suggested that even after metabolic activation of the test chemicals this cell system does not provide an adequately sensitive test for mutagenesis.

In additional experiments further information was obtained which showed enhancement of transformation by hydrazine and 1,2-dimethylhydrazine of cells exposed to irradiated virus. Significant enhancement occurred in these in vitro tests only when the cells were exposed to the chemical 24 hours prior to virus infection. (KT/AW)

RESEARCH OBJECTIVES:

- a. Determine whether one of seven herpes simplex virus (HSV) type 2 temperature sensitive (ts) mutants will be a more sensitive indicator of viral-induced cell transformation than UV-damaged wild type virus.
- b. Determine whether any of the following chemicals are mutagenic in THO cells following metabolic activation by the host mediated assay:

dimethylnitrosamine  
hydrazine  
monomethylhydrazine  
1,2-dimethylhydrazine  
1,1-dimethylhydrazine  
JP-5  
JP-10  
RJ-4  
RJ-5  
Ethylmethane sulfonate (direct-acting control)

STATUS OF THE RESEARCH EFFORT: ACCOMPLISHMENTS.

A. Transformation of 3T3 Cells by Temperature Sensitive Mutants of Herpes Simplex Virus Type 2.

A series of seven HSV-2 ts mutants were received for testing. Stocks of each ts mutant were prepared using standard methods in HEp-2 cells, and were titrated by plaque assay in HEp-2 cells at both permissive temperature ( $34^{\circ}\text{C}$ ) and restrictive temperature ( $38^{\circ}\text{C}$ ). The stocks were vialled in 2 ml aliquots and stored frozen at  $-70^{\circ}\text{C}$ .

The transformation assays were carried out in Swiss albino mouse 3T3 cells obtained originally from the American Type Culture Collection (Rockville, MD). The culture medium was Dulbecco's Modified Eagle's medium (DME) containing 10% fetal bovine serum or 10% newborn calf serum, 0.1% sodium bicarbonate, 100 units of penicillin per ml and 100 ug of streptomycin per ml.

Two methodologies were used to determine transformation by the mutant viruses. First, cells in suspension were infected with virus, then plated. 3T3 cells ( $1 \times 10^6$  per 1 dram vial) were infected at a multiplicity of either 1, 5, or 10 plaque forming units (pfu) per cell. The mixtures were incubated at  $38^{\circ}\text{C}$  with shaking for one hour. The contents of each vial was distributed between four 60 mm tissue culture dishes containing 4 ml of media, so that each plate was seeded with 250,000 cells. The plates were incubated at  $38^{\circ}\text{C}$  for 4-5 weeks and the media were renewed at weekly intervals. The plates were then stained with Wright's stain and examined microscopically for transformed foci and scored for the percentage of the monolayers surviving viral CPE. Sufficient plates were included in each experiment so that from  $3 \times 10^6$  to  $5 \times 10^6$  cells were infected at each multiplicity, which also included the non-infected controls.

The results of these experiments are shown in Table 1. Virus Ag (293) showed promise because at Moi=1 100% of the monolayers remained intact (minimum virus leak) and 31.6 foci were registered per  $10^6$  cells. This compares with about 4 foci per  $10^6$  cells for UV-inactivated wild type virus. This mutant is currently being employed in assays testing hydrazine and 1,2-dimethylhydrazine for transformation enhancement.

The second method of testing the ts mutants for transformation was to infect cells after attachment in a monolayer. 250,000 3T3 cells were seeded in each of a series of plates and the cells allowed to attach. From 12 to 20 plates were infected with each virus concentration (Moi=1, 5, and 10). Upon inoculation of the virus the previous media were removed, the virus inoculum added, and the plates were incubated at  $38^{\circ}\text{C}$  with periodic shaking for one hour. Fresh

media were then added, the plates incubated for 4-5 weeks, stained, and examined microscopically for monolayer survival and focus formation. The results are shown in Table 2. Again, the best results were obtained with Ag (293) which showed the greatest level of transformation with minimum leak. In comparing the results in Table 1 and Table 2, it appears that using Ag (293) at an Moi of 1 and infecting cells in suspension provides the optimum combination and should, in fact, be a superior test system over irradiated wild type virus for detecting chemical enhancement of herpes virus-induced transformation.

#### B. Mutagenesis in THO Cells Following Metabolic Activation.

The THO cells utilized in this study are mutant cells derived from the mouse Balb 3T3 line. They lack HPRTase function. Revertants can be selected in HAT medium as they grow to form colonies. These experiments were designed to determine if THO cells can be used as a mutagen screening test after the mutagens have been metabolically activated in the host mediated assay. In a previous report we showed that THO registered direct mutagenic activity only at a very low level.

The experiments were carried out in the following manner:  $4 \times 10^6$  THO cells, contained in media (Eagle's Basal Medium supplemented with 10% newborn calf serum, 0.22% sodium bicarbonate, L-glutamine and 100 units of penicillin per ml and 100 ug of streptomycin per ml) were injected intraperitoneally in 16-18 g female Balb/c mice. Immediately following, the mice received a subcutaneous injection of the potentially mutagenic chemical at a dose of 10 ml/kg. The cells remained in the peritoneal cavity for 6 hours or until the animal died of chemical toxicity whereupon the cells were harvested by injecting 3 ml of media, then removing the cells and media from the peritoneal cavity with a syringe and perforated needle. The cells in these harvests were counted in a hemocytometer (excluding erythrocytes) and plated in media in  $75 \text{ cm}^2$  flasks. The following day the media were renewed, then incubated for an expression time of 7 to 9 days. At this point, the cells were again trypsinized and seeded into HAT medium. After 7 to 10 days the colonies were stained and counted. The cell numbers were not counted at the time of seeding into HAT which accounts for the relatively large mutation frequencies shown on Table 3, but, rather, the viable recovered cells were used for this calculation.

The results of two experiments are shown on Table 3. Even though these experiments could only be regarded as preliminary, the conclusion indicated that the THO system is not a good mammalian cell test system, even when a known direct acting control (EMS), and a known mutagen requiring activation (dimethylnitrosamine) are included in the tests.

C. Further Studies on Transformation Enhancement Using UV-Irradiated Virus.

Initial studies in this laboratory suggested that hydrazine and 1,2-dimethylhydrazine (SDMH) enhanced viral transformation in a system utilizing 3T3 cells and UV-inactivated HSV-2. These results prompted further experimentation which we have carried out this year.

Employed in these assays was the 333 strain of HSV-2. The virus was irradiated in the following manner: 1.5 ml of virus stock was distributed evenly in a 60 mm plastic petri dish and, with the lid removed, exposed to 60 ergs/s/mm<sup>2</sup> of UV light for 2 minutes, agitated for 30 seconds; then irradiated for an additional 2 minutes.

Cells (250,000 per plate) were exposed to various concentrations of hydrazine or SDMH contained in 5 ml of media. Exposure of the cells to the chemicals was for 24, 6 or 2 hours prior to addition of the virus or for 24 hours beginning at 2, 6 or 24 hours after addition of the virus. Replicate plates were included in the tests which received chemical only. These cells were trypsinized and re-plated to determine the amount of chemical cytotoxicity. Thus, the surviving fraction could be calculated.

The plates containing cells exposed to both virus and chemical were incubated for four weeks with weekly media changes. They were then stained and examined microscopically for transformed foci. The results are shown on Tables 4 and 5.

It can be seen that both hydrazine and SDMH enhanced virus mediated transformation. Enhancement was time-dependent occurring only at -24 hours (addition of the chemical 24 hours before the virus).

PUBLICATIONS:

We have not yet published this work.

PROFESSIONAL PERSONNEL ASSOCIATED WITH THE RESEARCH EFFORT:

1. F. Brent Johnson, Ph.D., principal investigator.
2. Joyce Baker, Ph.D., research technician.

INTERACTIONS:

Participation in and presentation of a report at the Review of Air Force Sponsored Basic Research in Environmental Protection, Toxicology, and Electromagnetic Radiation Bioeffects, 15-17 January 1980, San Antonio, Texas.

NEW DISCOVERIES STEMMING FROM THE RESEARCH EFFORT:

1. That HSV-2 ts A<sub>8</sub> (293) represents a likely candidate for further transformation-enhancement studies.
2. That THO cells are a disappointingly insensitive system for mutagen testing.
3. Further data were obtained on enhancement of HSV-2 transformation by hydrazine and 1,2-dimethylhydrazine.

Table 1

Transformation of 3T3 Cells in Suspension by HSV-2 ts Mutants

Mutant	Non-infected Control		Moi=1		Moi=5		Moi=10	
	Surviving Monolayer		Surviving Monolayer		Surviving Monolayer		Surviving Monolayer	
	Foci/10 <sup>6</sup> cells							
A <sub>8</sub> (293)	100%	0	100%	31.6 ± 3.8	25%	29.25 ± 12.3	<<10%	2± 1.4
B <sub>5</sub> (155)	100%	0	100%	2.8 ± 3.3	75%	4.6 ± 2.9	50%	7±2.5
C <sub>2</sub> (116)	100%	0	100%	3.4 ± 1.5	25%	4.25 ± 3.0	10%	3.5 ± 0.7
E <sub>7</sub> (239)	100%	0	50%	1.67 ± 1.2	25%	2.33 ± 0.6	10%	0
F <sub>3</sub> (2C9)	100%	0	25%	6.4 ± 2.9	10%	1.75 ± 1.7	0	0
D <sub>6</sub> (144)	100%	0	<10%	1.2 ± 1.6	<<10%	0.25 ± 0.5	0	0
G <sub>4</sub> (148)	100%	0	10%	3.0 ± 0.7	<10%	0.5 ± 0.57	0	0

Table 2

## Transformation of 3T3 Cells on Monolayers by HSV-2 ts Mutants

Mutant	Non-infected Control		MoI=1		MoI=5		MoI=10	
			Surviving Monolayer		Surviving Monolayer		Surviving Monolayer	
	Surviving Monolayer	Foci/10 <sup>6</sup> cells	Monolayer	Foci/10 <sup>6</sup> cells	Monolayer	Foci/10 <sup>6</sup> cells	Monolayer	Foci/10 <sup>6</sup> cells
A <sub>8</sub> (293)	100%	0	100%	8.5 ± 2.1	25%	3.5 ± 0.7	<10%	0
B <sub>5</sub> (155)	100%	0	100%	1.75 ± 1.3	50%	6.0 ± 2.0	10%	5 ± 5.7
C <sub>2</sub> (116)	100%	0	10%	4.5 ± 0.7	<10%	0.5 ± 0.7	0	0
E <sub>7</sub> (239)	100%	0	75%	0.67 ± 0.6	50%	2 ± 1	25%	3.67 ± 2.5
F <sub>3</sub> (209)	100%	0	10%	4.0 ± 1.0	0	0	0	0
D <sub>6</sub> (144)	100%	0	<10%	1.0 ± 1.0	0	0	0	0
G <sub>4</sub> (148)	100%	0	50%	3.0 ± 2.0	10%	0	0	0

Table 3

Mutagenicity of Test Chemicals in THO Cells Following Host-Mediated Metabolic Activation

## (Experiment 1)

<u>Chemical</u>	<u>Time of Activation (min.)<sup>a</sup></u>	<u>No. Cells Recovered per ml.</u>	<u>No. Cells Seeded<sup>b</sup></u>	<u>No. Viable Cells Re-covered</u>	<u>No. of Revertant Cells<sup>c</sup></u>	<u>Mutation Frequency<sup>d</sup></u>
dimethylnitrosamine	180	230,000	345,000	37,375	207	5.5 x 10 <sup>-3</sup>
hydrazine	10	16,000	32,000	5,728	19	3.3 x 10 <sup>-3</sup>
monomethylhydrazine	10	52,000	104,000	18,754	18	1.0 x 10 <sup>-3</sup>
1,2-dimethylhydrazine	360	326,000	489,000	19,723	70	3.5 x 10 <sup>-3</sup>
1,1-dimethylhydrazine	25	100,000	150,000	26,500	123	4.6 x 10 <sup>-3</sup>
control	360	2,900,000	4,000,000	564,000	2250 est. <sup>e</sup>	4.0 x 10 <sup>-3</sup> est.

## (Experiment 2)

ethylmethane sulfonate	40	406,000	1,015,000	36,337	394	1.1 x 10 <sup>-2</sup>
JP-5	360	61,000	12,200	3	0	<0.33
JP-10	360	986,000	197,200	39	17	4.4 x 10 <sup>-1</sup>
RJ-4	360	102,000	81,600	65	159	2.4
RJ-5	360	176,000	105,600	21 est.	34	<1.6
control	360	138,000	165,600	66	226	3.4

<sup>a</sup> Time of in vivo exposure of the cells to activated chemical.

<sup>b</sup> No. of cells seeded in normal media at the beginning of the expression time.

<sup>c</sup> No. of colonies formed in HAT media.

<sup>d</sup> Mutation Frequency = No. of mutant cells divided by the no. of viable cells.

<sup>e</sup> Estimate

Table 4

Enhancement of HSV-2 wt Transformation by Hydrazine

<u>Time of car- cinogen addition (h)</u>	<u>Hydrazine (<math>\mu</math>l/ml)</u>	<u>Total colonies per 5000 cells</u>	<u>Surviving fraction</u>	<u>Transformed foci/10<sup>6</sup> cells</u>	<u>Transform- ation frequency</u>	<u>Enhancement ratio</u>
-24	0.05	11	0.0045	9	2000	1093
	0.01	112	0.0460	10	217	119
	0.005	267	0.1097	7	64	35
	0.001	767	0.3150	7	22	12
	0.0	2435	1.00	3	1.83	1
	0.05	53	0.0222	4	180	98
	0.01	61	0.0256	3	117	64
	0.005	1055	0.4423	3	7	4
	0.001	1215	0.5094	3	6	3
	0.0	2385	1.00	2	1.83	1
-6	0.05	607	0.2559	2	8	4
	0.01	1498	0.6315	5	8	4
	0.005	1762	0.7428	1	1.3	1
	0.001	1445	0.6092	1	1.6	1
	0.0	2372	1.00	4	1.83	1
	0.05	522	0.1813	0	-	-
	0.01	759	0.2635	0	-	-
	0.005	1086	0.3771	2	5	3
	0.001	1424	0.4944	4	8	4
	0.0	2880	1.00	1	1.83	1
+2	0.05	1206	0.4230	6	14	8
	0.01	1336	0.4686	5	11	6
	0.005	1473	0.5167	2	4	2
	0.001	1874	0.6573	3	5	3
	0.0	2851	1.00	1	1.83	1
	0.05	920	0.3199	0	-	-
	0.01	1514	0.5264	3	6	3
	0.005	1774	0.6168	1	1.6	1
	0.001	2024	0.7038	3	4	2
	0.0	2876	1.00	0	1.83	1
+6	0.05	1206	0.4230	6	14	8
	0.01	1336	0.4686	5	11	6
	0.005	1473	0.5167	2	4	2
	0.001	1874	0.6573	3	5	3
	0.0	2851	1.00	1	1.83	1
	0.05	920	0.3199	0	-	-
	0.01	1514	0.5264	3	6	3
	0.005	1774	0.6168	1	1.6	1
	0.001	2024	0.7038	3	4	2
	0.0	2876	1.00	0	1.83	1

Table 5

## Enhancement of HSV-2 wt Transformation by SDMH

Time of car- cinogen addition (h)	SDMH ( $\mu$ g/ml)	Total colonies per 5000 cells	Surviving fraction	Transformed foci <sup>1</sup>	Transform- ation frequency	Enhancement ratio
	5.0	55	0.0195	7	359	433
	1.0	107	0.0379	1	26	31
	0.5	112	0.0396	1	25	30
-24	0.5	111	0.0393	2	51	61
	0.1	165	0.0584	1	17	20
	0.05	2825	1.00	1	1	1
	0					
	5.0	795	0.2689	3	11	13
	1.0	878	0.2970	1	3	4
	0.5	1902	0.6434	2	3	4
	0.1	1521	0.5145	3	6	7
	0.05	983	0.3325	1	3	4
	0	2956	1.00	1	1	1
- 6	5.0	1137	0.4227	2	5	6
	1.0	878	0.3264	2	6	7
	0.5	885	0.3290	3	9	11
	0.1	1825	0.6784	2	3	4
	0					
- 2	0.05	2667	0.9914	1	1	1
	0	2690	1.00	1	1	1
	5.0	1154	0.4473	1	2	2
	1.0	1336	0.5178	1	2	2
	0.5	1497	0.5802	1	2	2
	0.1	2180	0.8450	0	-	-
+ 2	0.05	2289	0.8872	0	-	-
	0	2580	1.00	1	1	1
	5.0	-	-	1	-	-
	1.0	1078	0.4157	2	5	6
	0.5	-	-	1	-	-
+ 6	0.1	1142	0.4404	1	2	2
	0.05	1278	0.4929	1	2	2
	0	2593	1.00	0	-	-

Table 5 (cont.)

## Enhancement of HSV-2 wt Transformation by SDMH (cont.)

<u>Time of car-</u> <u>cinogen</u> <u>addition (h)</u>	<u>SDMH</u> <u>(<math>\mu</math>g/ml)</u>	<u>Total</u> <u>colonies</u> <u>per 5000 cells</u>	<u>Surviving</u> <u>fraction</u>	<u>Transformed</u> <u>foci</u>	<u>Transform-</u> <u>action frequency</u>	<u>Enhancement</u> <u>ratio</u>
<u>+24</u>	5.0	1391	0.4627	1	2	2
	1.0	1810	0.6021	1	2	2
	0.5	1997	0.6643	1	2	2
	0.1	2160	0.7186	3	4	5
	0.05	-	-	2	-	-
	0	3006	1.00	1	1	1